REMARKS

Claims 76-77, 81, and 83-127 are pending. In this Response, applicants amend Claims 76, 77, 103, and 116, and cancel Claims 82 and 128. The claim amendments do not introduce any new matter.

Obviousness-Type Double Patenting

The Examiner maintains a rejection of Claims 76-77 and 81-128 on the grounds of non-statutory obviousness-type double patenting over Claims 73-78, 80 and 85-90 of co-pending Application Serial No. 10/996,570. Applicants cancel Claims 82 and 128, thereby rendering their rejection moot. Applicants will file an appropriate terminal disclaimer if necessary once the Examiner finds allowable claims.

Rejection of Claims under 35 U.S.C. §101

The Examiner rejects Claims 76-77 and 81-128 under 35 U.S.C. §101. Applicants cancel Claims 82 and 128, thereby rendering their rejection moot. Applicants assert that claim amendments overcome the rejection of the pending claims and request its withdrawal.

The Examiner asserts that the claims are directed to naturally occurring compositions, a non-statutory subject matter (Office Action, page 4). Applicants amend the claims to clarify that the claimed compositions are obtained by an extracorporeal process and are not naturally occurring in human or animal body. The amendments are supported throughout the specification, as filed, for examples, on page 1, second paragraph.

35 U.S.C. §101 provides:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Applicants respectfully assert that pending claims are directed to subject matter in the statutory class "composition of matter" provided under 35 U.S.C. §101. Under 35 U.S.C. §101, nonnaturally occurring subject matter – "a product of human ingenuity – having a

distinctive name, character [and] use" is patentable. *See* MPEP 2105, citing *Diamond v. Chakrabarty*, 447 U.S. 303 (1980). Applicants assert that the compositions recited in the claims are nonnaturally occurring, are produced by an extracorporeal artificial process, and have distinctive name, character and therapeutic use, as discussed, for example, in the specification of the present application, on page 1, first paragraph. Accordingly, the claimed compositions are patentable subject matter under 35 U.S.C. §101.

Applicants do not claim naturally occurring compositions as an embodiment of their claimed invention and do not attempt to exclude others from the use of biological fluids or HDL particles naturally produced in a body of a human or an animal. Accordingly, Applicants respectfully assert that allowing pending claims to issue would not violate the principles of 35 U.S.C. §101. While Congress meant 35 U.S.C. §101 to exclude non-statutory items from even temporary monopolization by patent, even the claims reciting non-statutory items should be allowed to issue when they do not prohibit all uses of the non-statutory items. *In re Bernhart and Fetter* 163 U.S.P.Q. 611, 616 (1969). Here, allowing pending claims would not exclude others from the use of the compositions naturally produced in a body of a human or an animal.

The Examiner asserts that the claimed compositions contain pre-β HDL and are naturally produced in the liver of the animals (Office Action, page 4). Applicants respectfully bring to the Examiner's attention that pending claims do not recite pre-β HDL as a limitation. The claims, as currently amended based on previously presented claims, are directed to compositions formed by an extracorporeal process comprising exposing a biological fluid comprising high density lipoprotein particles to a lipid removing agent. The compositions obtained by the extracorporeal process comprise particle derivative of the high density lipoprotein particles having apolipoprotein A-1 and at least one of apolipoprotein C-III, apolipoprotein D or apolipoprotein E, and having a lower content of at least one of the phospholipids or cholesterol than the high density lipoprotein particles in the biological fluid prior to exposure of the biological fluid to the lipid removing agent. Applicants assert that the particle derivatives recited in the claims are not pre-β HDL and are not produced "in the liver of animals via the mechanisms of lipases or cholesteryl ester acceptors," contrary to what the Examiner asserts on page 4 of the Office Action.

Naturally occurring pre-β HDL is formed during reverse cholesterol transport from lipid free apoA-I with efflux of cellular cholesterol. *See* Sviridov *et al.*, "Dynamics of Reverse Cholesterol Transport: Protection against Atherosclerosis," *Atherosclerosis*, v. 161: 245-254 (2002) ("Sviridov"), Figure 1, cited by the Examiner in the Final Office Action. ApoA-I is the only protein component of pre-β_1 and pre-β_2 HDL. *See*, for example, Fielding and Fielding, "Molecular Physiology of Reverse Cholesterol Transport," *Journal of Lipid Research*, v. 36:211-228 ("Fielding"), pages 214-215 (Exhibit A). In contrast, particle derivatives of the currently pending claims comprise, as their protein components, apolipoprotein A-1 and at least one of apolipoprotein C-III, apolipoprotein D or apolipoprotein E as their protein components. For at least this reason, the particle derivatives recited in the claims are different from naturally occurring pre-β HDL.

As the evidence provided herein in the form of scientific articles supports, delipidation processes naturally occurring in the human body during reverse cholesterol transport (RCT) do not result in the claimed compositions. The Examiner discusses RCT as disclosed in Sviridov, for example, in Figure 1 and on pages 247-249 on page 8 of the Office Action. Small, discoid, lipid-poor pre-β_1 HDL particles serve as an initial acceptor of cellular cholesterol and are transformed into pre-β_2 HDL upon accumulation of cholesterol. As discussed above, both pre-β HDL species contain apoA-1 as their only protein component and are therefore distinct from HDL particle derivatives recited in the claims for at least this reason. Esterification of cholesterol by lecitihin:cholesterol acyltransferase (LCAT) in pre-β_2 HDL results in spherical α HDL particles, the form in which most HDL is present in plasma, which continue to acquire cholesterol and increase in size. *See* Sviridov, Figure 1; Kunitake *et al.* "Interconversion Between Apolipoprotein A-I-Containing Lipoproteins of Pre-beta and Alpha Electrophoretic Mobilities," *Journal of Lipid Research*, v. 33: 1807-1816 (1992) ("Kunitake"; cited by the Examiner in the Final Office Action), Figure 10; Fielding, page 215. When the largest of α HDL particles, α3 HDL, are remodeled by hepatic lipase (HL), cholesteryl ester transfer proteins (CETP), and phospholipid transfer proteins (PLTP), lipid-free apoA-I dissociates. *See* Sviridov, Figure 1 and page 247.

Therefore, contrary to what the Examiner states on page 4 of the Office Action, the action of lipases and cholesteryl ester transfer acceptors does not result in pre-β HDL, but, rather, it results in removal of apoA-I from spherical plasma HDL and production of lipid-free apoA-I. Pre-β HDL is subsequently produced from the lipid-free apoA-I via efflux of cellular cholesterol, and not from α3-HDL by HL, CETP and PLTP. *See* Sviridov, Figure 1. In contrast, the extracorporeal process recited in the claims produces HDL particle derivatives that possess apolipoprotein composition similar to that of naturally occurring particles. See February 8 Declaration of Mr. Hasibullah Akeefe ("Declaration"), of record in the present application, item 7. To re-state the foregoing, naturally occurring HDL remodeling processes not only lower HDL lipid content, but also changes apolipoprotein composition of the HDL particles being remodeled, such as by removal of apoA-I. In contrast, as stated in the Declaration, "particle derivatives [recited in the claims] ... inherently possess apolipoprotein composition similar to that of naturally occurring [HDL] particles" but lower lipid content than the naturally occurring HDL particles. This is a distinctive characteristic of the claimed compositions.

The claimed compositions are "a product of human ingenuity," as discussed in MPEP 2105, because they are obtained by nonnatural extracorporeal process, possess at least one characteristic distinctive from a naturally occurring biological fluid, and have a therapeutic use. In view of the amendments and the foregoing arguments, Applicants respectfully assert that the claimed compositions are patentable subject matter under 35 U.S.C. §101.

Rejection of Claims under 35 U.S.C. §102

Kunitake

The Examiner rejects Claims 76-77 and 81-128 under 35 U.S.C. §102(b) as anticipated by Kunitake. Kunitake discloses a study monitoring interconversion between pre-β HDL and α-HDL in plasma and upon exposure of isolated HDL to cells. Kunitake discloses isolation of lipoproteins, incubation of plasma and plasma components, and incubation of pre-β HDL and α HDL with cholesterol-loaded cells (see pages 1808 and 1809 of Kunitake). The Examiner asserts that Kunitake teaches all elements of Claims 76-77 and 81-128.

Applicants cancel Claims 82 and 128, thereby rendering their rejection moot. Applicants request withdrawal of the rejection of the pending claims in view of the claim amendments and arguments presented in the section "*Rejection of Claims under 35 U.S.C. §101.*" The claimed compositions are obtained by nonnatural extracorporeal process and are distinctive from naturally occurring compositions. Kunitake teaches compositions that are obtained by naturally occurring processes and do not comprise at least one limitation of the pending claims, a particle derivative of high density lipoprotein particles. Accordingly, Kunitake fails to teach all elements of the claims and fails to anticipate the claims. In view of the amendments and the foregoing arguments, Applicants request withdrawal of the rejection of claims under 35 U.S.C. §102(b) as anticipated by Kunitake.

Sviridov

The Examiner rejects Claims 76-77 and 81-128 under 35 U.S.C. §102(b) as anticipated by Sviridov. Sviridov is a review article regarding anti-atherogenic function of HDL and its metabolism in the human body. On page 8 of the Office Action, the Examiner appears to assert that the pending claims read on naturally occurring HDL species discussed in Sviridov on page 248, and schematically shown in Figure 1 on the same page.

Applicants cancel Claims 82 and 128, thereby rendering their rejection moot. Applicants request withdrawal of the rejection of the pending claims in view of the claim amendments and arguments presented in the section "*Rejection of Claims under 35 U.S.C. §101.*" The claimed compositions are obtained by nonnatural extracorporeal process and are distinctive from naturally occurring compositions. Sviridov teaches compositions that are obtained by naturally occurring processes and do not comprise at least one limitation of the pending claims, a particle derivative of high density lipoprotein particles. Accordingly, Sviridov fails to teach all elements of the claims and fails to anticipate the claims. In view of the amendments and the foregoing arguments, Applicants request withdrawal of the rejection of claims under 35 U.S.C. §102(b) as anticipated by Sviridov.

Barrans

The Examiner rejects Claims 76-77 and 81-128 under 35 U.S.C. §102(b) as anticipated by Barrans et al. "Hepatic Lipase Induces the Formation of Pre-β1 High Density Lipoprotein (HDL) from Triacylglycerol-rich HDL2," *The Journal of Biological Chemistry*, v. 269: 11572-11577 (1994) ("Barrans"). Barrans describes perfusion of native tryacylglycerol-enriched human HDL2 though the rat liver and incubation of these HDL2 particles in vitro with rat hepatic lipase. *See* Barrans, Abstract, pages 11573-11574. Barrans reports the formation of pre-β1 HDL in both disclosed experiments. *Id.* The Examiner asserts that Barrans teaches all elements of the pending claims.

Applicants cancel Claims 82 and 128, thereby rendering their rejection moot. Applicants request withdrawal of the rejection of the pending claims in view of the claim amendments and arguments presented in the section "*Rejection of Claims under 35 U.S.C. §101.*" The claimed compositions are obtained by nonnatural extracorporeal process and are distinctive from naturally occurring compositions. Barrans teaches compositions that are obtained by processes naturally occurring in liver. The compositions disclosed in Barrans and do not comprise at least one limitation of the pending claims, a particle derivative of high density lipoprotein particles. Pre-β1 HDL obtained by Barrans is distinct from the particle derivatives of high density lipoprotein particles at least due to the differences in protein composition. Accordingly, Barrans fails to teach all elements of the claims and fails to anticipate the claims. In view of the amendments and the foregoing arguments, Applicants request withdrawal of the rejection of claims under 35 U.S.C. §102(b) as anticipated by Barrans.

CONCLUSION

The foregoing is submitted as a full and complete response to the Final Office Action mailed June 12, 2007. No additional fees are believed due, however, the Commissioner is hereby authorized to charge any deficiencies which may be required or credit any overpayment to Deposit Account Number 11-0855.

Applicants assert that the claims are in condition for allowance and respectfully request that the application be passed to issuance. If the Examiner believes that any informalities remain in the case that may be corrected by Examiner's amendment, or that there are any other issues which can be resolved by a telephone interview, a telephone call to the undersigned agent at (404) 815-6102 or to Dr. John McDonald at (404) 745-2470 is respectfully solicited.

Respectfully submitted,

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review

Molecular physiology of reverse cholesterol transport

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Abstract Reverse cholesterol transport (RCT) is the pathway by which peripheral cell cholesterol can be returned to the liver for catabolism. Evidence of specific functions for molecular structures within individual plasma lipoprotein species has rapidly accumulated from recent studies using molecular and cellular physiology techniques. The removal of cholesterol from cells, like its delivery, appears to be specific and well regulated. Although further research will be needed, RCT can now be understood in molecular terms.—**Fielding, C. J., and P. E. Fielding.** Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* 1995. 36: 211–228.

Supplementary key words lipid transfer proteins • high density lipoproteins • apolipoprotein A-I • lecithin:cholesterol acyltransferase • HDL binding proteins

INTRODUCTION

Peripheral (nonhepatic) cells obtain their cholesterol from a combination of local synthesis and the uptake of preformed sterol from low and very low density lipoproteins (LDL and VLDL). Reverse cholesterol transport (RCT) is the opposing movement of cholesterol from peripheral cells through the plasma compartment to the liver (1). Here this cholesterol can be recycled in newly secreted plasma lipoproteins, but part appears in the bile as free cholesterol or (after degradation) as bile acids. Cholesterol in bile, reabsorbed in the intestine and secreted along with dietary cholesterol into the intestinal lymph, reappears in the plasma as chylomicrons. Chylomicron remnants, retaining most of their original cholesterol content after lipolysis, are rapidly cleared by the liver. This cholesterol then becomes available again for cellular metabolism or recycling as plasma lipoproteins (**Fig. 1**).

Without input of energy, the concentration of cholesterol in different tissues, and in extracellular lipoproteins, would come to a true equilibrium. Cholesterol would continue to exchange between the different pools, but there would be no net transport between them. It is now quite clear that active transport mechanisms of several kinds do perturb this simple equilibrium in vivo. As a result the homeostasis of cholesterol is both metastable and dynamic. The delivery of cholesterol into cells by receptor-mediated endocytosis represents one such perturbing force. The de novo synthesis of cholesterol is another. Because transport along the pathways shown in Fig. 1 is not at a true equilibrium, individual rates are kept consistent by continuous input from positive and negative controls. Changes in cholesterol demand can then be rapidly accommodated and finely controlled, but a system like this is very sensitive to small changes in substrate concentrations and rates.

Glomset (2) was the first to recognize that RCT involved the active transport of cholesterol, as cellular free cholesterol became converted to its insoluble ester outside the cell. The enzyme concerned, lecithin:cholesterol acyltransferase (LCAT), appears in all modern accounts of RCT. LCAT, as part of plasma high density lipoprotein (HDL), increases cholesteryl ester in this lipoprotein fraction. Two lipid transfer proteins contribute to further HDL remodelling. Unlike an enzyme, these proteins are energy-independent, and they must work down preexisting concentration gradients. A phospholipid transfer protein (PLTP) supplies lecithin to HDL (3) and may be a factor in other remodelling reactions. A cholesteryl ester transfer protein (CETP) can move cholesteryl ester made by LCAT to other lipoproteins, particularly triglyceride-rich lipoproteins and LDL (4). HDL triglyceride can then be catabolized by the extracellular hepatic triglyceride lipase (1). Finally, lipoprotein cholesterol is removed by

Abbreviations: RCT, reverse cholesterol transport; LCAT, lecithin: cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; LpA-I, HDL containing only apoA-I as protein; LpA-I,A-II, HDL containing both apoA-I and apoA-II as protein; apoE, apolipoprotein E; FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; PLTP, phospholipid transfer protein; CETP, cholesteryl ester transfer protein; SCP-2, sterol carrier protein-2; FABP, fatty acid-binding protein; kBa, kilobase. Protein sequences use the single letter code.

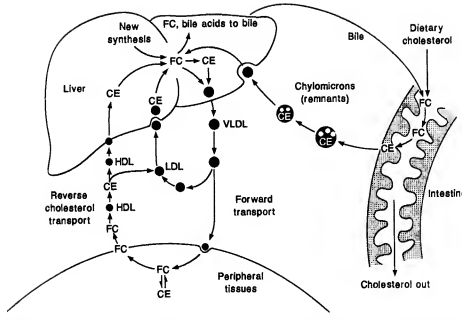

Fig. 1. Major cholesterol transport pathways between liver and peripheral tissues. FC, free cholesterol; CE, cholesteryl ester; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. Arrows indicate the direction of net transport; for clarity nonproductive exchanges of FC between lipid surfaces are not illustrated.

the liver via several different mechanisms (5). Only tissues that actively interiorize or synthesize cholesterol contribute to RCT as defined above. Red blood cells, which do neither, are only a passive buffer of transient changes in plasma free cholesterol concentration.

Because RCT is part of a dynamic equilibrium, many different factors affect the rate constants of each step: efflux, esterification, transfer, and clearance (1). Additionally, every lipid and protein component of HDL particles is itself in independent equilibrium with other lipoproteins and/or membrane surfaces. What we now know about this very complex system comes mainly from two types of investigation. Biochemical analysis of individual proteins active in RCT has been recently extended using molecular and immunobiological techniques. RCT has also been studied with kinetic and cell physiology approaches in complete systems such as plasma or lymph in the presence of vascular or other cells, and in mice in which individual factors within the RCT pathway were overexpressed or knocked out using transgenic techniques. Here these different kinds of information have been combined to give a molecular overview of the physiology of RCT.

PROTEINS ACTIVE IN THE RCT PATHWAY

Plasma membrane

Cholesterol is distributed asymetrically in the plasma membrane bilayer. In nucleated cells, most cholesterol is located on the inner leaflet of the plasma membrane bilayer. This distribution can be modified by changes in membrane fatty acid composition and headgroup (6, 7). Paradoxically, the major part of membrane sphingomyelin, which has a higher affinity for cholesterol than other major phospholipids in model systems, is normally in the outer leaflet of the bilayer. A model of lipid microdomains with differing cholesterol content has been described to account for the multiple cholesterol pools predicted from the complex kinetics of efflux of membrane sterol (8). To some extent this heterogeneity, while no doubt influenced by membrane lipid composition, may also be an inherent property of cholesterol in phospholipid bilayers (9). The localization of membrane proteins also defines small (1 μm diameter) microdomains in the plasma membrane (10). Finally, intracellular lipid transport proteins can modify cholesterol bilayer distribution (11) and the kinetics of efflux (12).

Several laboratories have identified HDL binding proteins on the plasma membranes of cells including fibroblasts, hepatocytes, adipocytes, and macrophages (13–18). Molecular masses of 80–180 kDa have been reported. An HDL binding protein of wide distribution (80–110 kDa in different tissues) may promote the efflux of free cholesterol to the medium while a larger protein (180 kDa) (18) present only in hepatocytes could be important for the interiorization of intact HDL particles.

The binding of HDL or isolated apolipoproteins to cell membrane HDL binding proteins shows rather broad apolipoprotein specificity, with apoA-I, apoA-II, and

apoA-IV all reported active. The functional domain in apoA-I was recently localized with monoclonal antibodies to the C-terminus of the mature protein (19). A localization generally consistent with this has been obtained using graded carboxypeptidase degradation (20). A homologous amino acid sequence in apoA-II appears to play the same role (21).

An HDL-binding protein has been recently cloned (22). Unexpectedly, its cDNA sequence predicts no obvious membrane-spanning sequence. Repeating amphipathic helical segments comparable to those present in apoA-I were found although the functional significance of these is unclear.

Structure of apolipoprotein A-I (apoA-I)

ApoA-I is present in at least one copy (and usually two to four copies) in each HDL particle of normal plasma. The preproapolipoprotein (267 aa), originates in liver and (in humans) also in the small intestine. It is secreted as a 249-aa proprotein that is rapidly cleaved by a plasma protease to generate the mature 243 aa polypeptide (23).

The primary sequence of apoA-I is shown in Fig. 2 (legend:refs. 24–25). ApoA-I consists mainly of 22-aa repeating segments, typically spaced with helix-breaking proline residues (26). In the presence of phospholipid these repeats form amphipathic alpha-helices, with charged residues facing the aqueous medium and nonpolar residues facing the acyl chains of the phospholipid bilayer (27). The structure may be further stabilized by charge-charge interaction between adjacent repeats. There is a high proportion of helix destabilizing amino acids (D, E, R, K, G) on the polar face of many of the repeats, possibly to facilitate their expansion and contraction on the HDL surface during metabolism.

ApoA-I is present in the plasma of even the lowest vertebrates. Comparison of human, rat, and chick apoA-I sequences (Fig. 2) shows that much of the total variability is concentrated in two of the repeats (human apoA-I residues 122–143 and 166–187, respectively) while the repeat between residues 144–165 is more highly conserved. Scoring hydrophobic, hydrophilic neutral, and positively and negatively charged residues, conservation among all three species is 85% of residues 144–165, and 38% and 43%, respectively, for residues 122–143 and

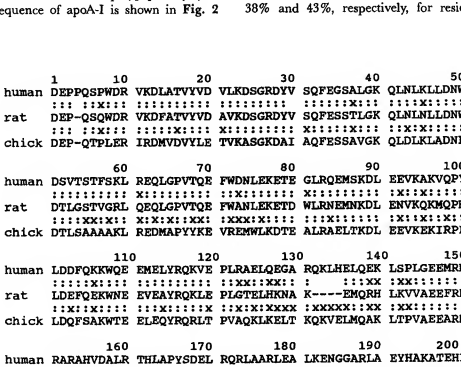

Fig. 2. Apolipoprotein A-I sequence in human, rat, and chicken. Sequences are from refs. 24 and 25. The single letter code is used; x, unlike-kind amino acids; :, like-kind or identical amino acids; –, absent.

166–187. For other repeats it varies between 65% and 81%. Conservation between species often identifies important structural and/or functional elements within the primary sequence. Charged residues in the variable repeats of apoA-I are not well conserved between species, although the ability to activate human LCAT is similar (28), which suggests that charge–charge interactions between adjacent amphipathic helices within residues 122–143 and 165–187 are not functionally important.

ApoA-I forms three types of stable structure with lipids: small lipid-poor complexes; flattened discoidal particles containing only polar lipids (phospholipid and cholesterol); and spheroidal particles containing both polar and nonpolar lipids.

Prebeta-1 HDL, "lipid-poor apoA-I". Plasma HDL normally contains 2–5% of small particles distinguished by a slow (prebeta-) electrophoretic mobility from the bulk of alpha-migrating HDL (29–31). The apparent molecular mass of these particles was 60–70 kDa in different studies, with a calculated diameter of 5–6 nm. The protein moiety contains only apoA-I. Lipid contents of 10–40% have been reported. Higher concentrations of prebeta-1 HDL are present in large vessel lymph (32, 33). Prebeta HDL were also found at increased relative concentration within the aortic intima (34, 35). Prebeta-1 HDL were completely lost from the HDL density range by ultracentrifugal flotation (31).

Lecithin and sphingomyelin were present at almost equal molar concentrations in prebeta-1 HDL, a proportion found otherwise only in the outer leaflet of the plasma membrane (36) (**Table 1**).

The conformation of apoA-I in prebeta-1 HDL differs from that in other HDL. Lipid-poor apoA-I includes a much lower alpha-helical content. Whether this is the cause or a result of the unusual phospholipid composition of these particles remains to be determined. A series of monoclonal antibodies recognizing different epitopes in apoA-I was raised in mice against delipidated apoA-I. A

TABLE 1. Sphingomyelin–lecithin ratio of prebeta-HDL

Fraction	Ratio
Plasma membrane outer leaflet[a]	1.00 ± 0.05
Prebeta-1 HDL	0.95 ± 0.14
Prebeta-2 HDL	0.46 ± 0.10
Prebeta-3 HDL	0.70 ± 0.22
HDL_{2b}	0.16 ± 0.02
HDL_{2a}	0.20 ± 0.02
HDL_3	0.20 ± 0.00

P. E. Fielding and T. Miida, unpublished data. Total phospholipid was extracted with chloroform–methanol from pooled gel fragments recovered from two-dimensional nondenaturing electrophoresis (ref. 29). Fractionation was carried out on silicic acid layers developed in chloroform–methanol–isopropanol–0.25% aqueous KCl–triethylamine 30:9:25:6:18 (v/v).

[a]From ref. 36.

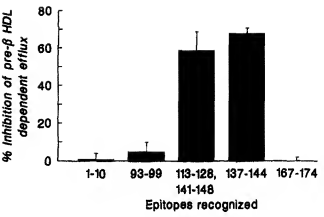

Fig. 3. Inhibition of cholesterol efflux from fibroblast monolayers in the presence of mouse monoclonal antibodies against human apoA-I. Each antibody is defined in terms of the amino acid residues recognized within the primary sequence of mature apoA-I. Inhibition is given in terms of the prebeta-HDL component of total efflux, which in these studies was 60% of total efflux.

continuous epitope recognizing residues 137–144 of the mature apoA-I polypeptide (~LQEKLSPL~) was detected only on prebeta-1 HDL (37). In the presence of this antibody the ability of prebeta-1 HDL to promote the efflux of cholesterol from cell membranes was significantly inhibited (**Fig. 3**). Small prebeta HDL were also uniquely susceptible to proteolytic cleavage by thrombin near residue 100 of apoA-I (38). Both regions mark beta-turns between repeating 22-residue amphipathic helical segments in the primary sequence. Other sequences in prebeta-1 HDL accessible to monoclonal antibodies include the N-terminus (residues 1–10) and residues 167–174. Prebeta-1 HDL did not react with a monoclonal antibody that recognized residues 93–99 in discoidal HDL (37). These data indicate a unique organization of apoA-I in lipid-poor HDL. These features are included in the model shown in **Fig. 4**.

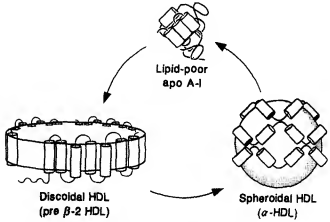

Fig. 4. ApoA-I structure in lipid-poor, discoidal, and spheroidal HDL. Models of particles containing three apoA-I in discoidal and spheroidal HDL are illustrated.

Prebeta-2 HDL, discoidal HDL. An HDL fraction (prebeta-2 HDL) present in plasma to the extent of 2–3% of particles contains three apoA-I per particle as the only protein (29). It is rich in lecithin and contains a smaller proportion of sphingomyelin and cholesterol (Table 1). Prebeta-1 and prebeta-2 differ mainly by their proportion of phospholipid, particularly lecithin. Cholesteryl ester was not detected in prebeta-2. As a result its lipid composition is similar to that of the HDL discs that accumulate in plasma when LCAT is absent or inhibited (39). The diameter of synthetic discoidal HDL varies with lipid content and the number of apoA-I polypeptides per particle, but their thickness remains remarkably constant. Sectional electron microscopy indicates that discoidal HDL are made of a single lipid bilayer (40) probably stabilized by protein at the periphery. Computer modelling of discoidal HDL suggests that the repeating 22-aa helices of apoA-I run from side to side of the disc (27, 41, 42). With 3.6 residues per turn and an advance of 0.54 nm, each repeating unit would occupy six turns taking up about 3.2 nm, similar to the thickness of a phospholipid bilayer (Fig. 4). Discoidal HDL, like lipid-poor prebeta HDL, are present at increased concentration in large vessel lymph (32, 33).

As in the case of prebeta-1 HDL, strong epitopes seem to be often located at beta-turns between adjacent repeating segments when prebeta-2 HDL reacts with monoclonal antibodies raised against delipidated apoA-I. Prebeta-2 HDL in plasma reacted strongly with a monoclonal antibody recognizing residues 93–99 (-VKAKVQP-) but not with that recognizing residues 137–144 in prebeta-1 HDL (37).

The C-terminal part of apoA-I may be of significance in phospholipid binding in discoidal HDL. Deletion of residues 212–243 by mutagenesis inhibited disc formation in one study (43), but shorter deletions within the same region did not (44).

Many different types of synthetic recombinant HDL discs have been prepared with two to four apoA-I per particle and a range of molecular masses from 120–400 kDa (45, 46). However, the conformation of apoA-I in these and native HDL discs has not been systematically compared. As in apoE (47), significant local tertiary structure may exist in apoA-I despite an overall low free energy of denaturation.

Spherical HDL. Most HDL in plasma are present as spherical particles (9–12 nm diameter) with alpha-migration in agarose electrophoresis. Three size subclasses predominate (HDL_3, HDL_{2a}, and HDL_{2b} in order of increasing size and lipid content) (48). Traces of larger and smaller species are often present (29, 31). Most HDL contain both apoA-I and apoA-II, the second major HDL protein. The proportion of total HDL containing only apoA-I (LpA-I) was 11–45% in different studies (49–51), a range probably depending, in part, on the technical details of separation. ApoA-I may stabilize HDL core cholesteryl esters (52). This probably involves an interaction between these lipids and the hydrophobic face of several apoA-I repeating subunits.

When monoclonal antibodies were raised against spheroidal HDL, most of them recognized discontinuous epitopes (53). This suggests that the organization of repeating sequences of apoA-I was disordered in spherical HDL compared to that in discs. Beta-turns from different apoA-I polypeptides may be in contact with each other on the surface of the sphere. Charge-charge interactions between adjacent repeats of the same polypeptide appear to be less important in spheroidal than in discoidal HDL (Fig. 4). A similar model of spherical HDL was suggested by Brasseur et al. (41).

The presence of smaller but still significant amounts of other apolipoproteins within HDL, together with variation in lipid composition and protein conformation, makes the number of spherical HDL species potentially very large. Many of the minor apolipoproteins, and the complexes they form with HDL, probably have no special role in RCT. Recent emphasis has focussed on the properties of spheroidal HDL that contain only apoA-I (two to four copies per particle) and apoA-II (one to two copies per particle) (54). Spherical HDL particles probably shrink and grow many times during their circulatory period (typically 2–3 days) during fasting and dietary lipid absorption (55) but there appear to be few differences in the conformation of apoA-I in large and small spherical HDL (56).

Structures of other HDL apolipoproteins implicated in RCT

Three other apolipoproteins show marked sequence homology to apoA-I, although present in much lower concentration in plasma. ApoA-IV, apoC-1, and apoE also have amphipathic helical repeating units (24, 26) and can activate LCAT in synthetic discoidal recombinants, although less effectively than apoA-I (57). These apolipoproteins may substitute for some of the functions of apoA-I when plasma apoA-I is very low or absent.

Lecithin:cholesterol acyltransferase (LCAT)

LCAT reacts with discoidal and spherical HDL, transferring the 2-acyl group of lecithin or phosphatidylethanolamine to the free hydroxyl residue of fatty alcohols, particularly cholesterol, to generate cholesteryl esters (retained in the HDL) and lysolecithin. In the complete absence of cholesterol, LCAT is a phospholipase, generating lysolecithin and free fatty acids. LCAT also catalyzes an acyl exchange between lecithin and lysolecithin; the effect is to redistribute 1- and 2-position fatty acids within lecithin (58, 59). The catalytic rate of LCAT is affected by the surface properties of individual lecithins, probably more so than on acyl chain length as such.

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Lecithins with widely different saturation and chain lengths dispersed in an inert phosphatidyl ether matrix have similar reaction rates (60).

The LCAT gene is present as a single copy (61). A 1.5-kBa hepatic LCAT mRNA codes for the full-length active protein in liver (62). A 7-kBa mRNA is also present but its translation product has not been identified. LCAT, like many other lipases and esterases, is a serine hydrolase, probably with a catalytic triad also including histidine and a glutamate or aspartate residue. The active-site serine residue has been identified by site-directed mutagenesis as ser_{181} (63). Immediately prior to and following ser_{181} are short sequences of hydrophobic amino acids predicted to form a beta sheet. Between residues 151 and 174, a 23-amino acid sequence is found that has marked sequence similarity to an amphipathic helical repeat in the C-terminus of apoE (64):

```
human LCAT   P150GQQEEYYRKLAGLVEEMHAAYG
                x x : : : x : x : : : : : : x : x : : : :
human apoE   P267- LVEDMQRQWAGLVEKVQAAVQ
```

This structure is absent in other serine-dependent lipases and esterases. The other active site residues have not yet been located. LCAT has free sulfhydryl groups at residues 31 and 184. Mutagenesis indicates that these are not required for activity; the classical inhibition of LCAT by sulfhydryl reagents (like that of many other "-SH enzymes") must therefore be the result of steric inhibition, and no -S-acyl intermediate is formed as part of the catalytic cycle (65, 66).

A high concentration of LCAT (relative to apoA-I) is found in a prebeta-migrating high molecular weight complex (prebeta-3 HDL) (67). This is likely to correspond to the LpA-I-only fraction of LCAT identified using immunoaffinity chromatography (68). Prebeta-3 is probably a complex between discoidal (prebeta-2) HDL and LCAT, with the addition, possibly transient, of other catalytic proteins such as CETP. A small amount of LCAT was always associated with LDL (67).

Cholesteryl ester and phospholipid transfer proteins (CETP, PLTP)

CETP has been cloned and sequenced and shows some sequence similarity to several other lipid-binding proteins (69). It catalyzes an exchange of neutral lipids, particularly triglyceride and cholesteryl esters, between all the major lipoprotein classes. In normal plasma net transport of cholesteryl ester by CETP depends mainly on the availability of suitable triglyceride-rich acceptor particles, not on CETP concentration (70). One laboratory has argued for a key role for a C-terminal "lipid-binding helix" and for the identity of binding sites for cholesteryl ester and triglyceride (71). Other data suggest a more complex mechanism, with cholesteryl ester and triglyceride requiring non-identical although probably partly overlapping C-terminal sequences, and a lipid-binding cleft to which hydrophobic sequences from several parts of the molecule contribute (72, 73).

The cDNA of PLTP, recently cloned from an endothelial cell library (74), predicts a protein sequence with 25% homology with CETP.

Proteins active in the hepatic uptake of cell-derived cholesterol

Three different mechanisms have been described for the clearance of LCAT-derived cholesteryl esters by hepatocytes. LDL receptor protein is active on hepatocytes in the endocytosis of LDL and VLDL remnant lipoproteins (75). Large apoE-rich HDL also have a high affinity for the LDL receptor (76) although the concentration of such particles in human plasma is very small (77).

Retroendocytosis of HDL by rat hepatocytes has been reported. Smaller but probably still spherical HDL are returned to the extracellular fluid (78). A disproportionate uptake of cholesteryl ester from HDL not involving lipoprotein apoE or the LDL receptor has been described (79). A recent study suggested that apoE bound to the hepatocyte surface might be required (80) but if so the detailed mechanism is not yet understood. The concentration of plasma HDL in apoE-knockout mice is low (81, 82) rather than increased, as might be expected if the clearance of HDL required apoE.

MOLECULAR MECHANISMS IN REVERSE CHOLESTEROL TRANSPORT

Cholesterol transfer from the cell surface

The peripheral tissues involved in RCT transfer cholesterol only in its unesterified form. Much the largest part of cholesterol from labeled peripheral cells is initially transferred to HDL (77) even though this fraction contains only about one quarter of total plasma free cholesterol. Two different hypotheses to explain cholesterol efflux have been proposed: that it is diffusion-based or that it is receptor-dependent.

Cholesterol transfer between synthetic lipid vesicles is evidently diffusional (83). As the aqueous solubility of cholesterol although low is certainly finite, diffusion must also contribute to total cholesterol efflux from cell membranes. The real question is, does diffusion account for the whole of cellular efflux, or only part? The most recent data suggest that efflux from nucleated cells reflects the existence of multiple plasma membrane cholesterol pools (12). If desorption from the membrane is rate-limiting, the complex kinetics of efflux can still be explained in terms of diffusion, if plasma membrane microdomains with different desorption rates are defined by different local lipid compositions (8). Can the concentration of cholesterol in the microdomains be regulated dynamically? Convincing evidence for this comes from the report

(12) that addition of the intracellular lipid-binding protein sterol carrier protein-2 (SCP-2) to cell membrane preparations significantly increased the fast component of sterol efflux from L-cell membranes. Another intracellular lipid transfer protein (L-FABP) increased the proportion of cholesterol on the outer leaflet of the plasma membrane in L-cells when these were transfected with L-FABP cDNA (11). In vitro, L-FABP did not increase fast-pool efflux from plasma membrane preparations (12). Efflux was not reduced in peroxisomal-deficient cells even though SCP-2 levels in these cells were much reduced (84). However higher molecular weight forms of SCP may be present and functional. Overall the evidence that sterol efflux can be modulated by intracellular lipid transfer proteins appears convincing, but further research will be needed to identify the roles of individual factors in the intact cell.

In the second hypothesis, cholesterol efflux follows from protein C kinase-mediated signalling initiated by the high-affinity binding of HDL to a cell-surface HDL binding protein (85, 86). Cholesterol is transferred to the cell surface from a pool of new synthesis in the endoplasmic reticulum. Desorption there to the medium is considered to be passive. While there is no doubt that signalling intermediates are present in vitro after HDL binding, the significance of the observation to normal physiology is not yet fully established. The phenomenon has been found mainly in lipid-loaded cells such as adipocytes and foam cells. Peripheral cells such as fibroblasts, when not cholesterol-loaded, showed little or no efflux via this pathway (85), yet HDL-dependent efflux from the plasma membrane, which normally contains 80–90% of cell cholesterol, is regularly observed in unloaded cells. The contribution of signal-mediated efflux to RCT in unloaded cells (which represent the vast majority of cells in vivo) is probably therefore quite small. Unloaded hepatocytes express HDL-binding proteins (15, 18). Possibly the normal role of HDL-binding proteins in these cells is in HDL internalization.

Neither hypothesis satisfactorily explains the specificity for HDL (particularly lipid-poor LpA-I-only, prebeta-1 HDL) as cholesterol acceptor now reported in a number of different studies. How good is the evidence that lipid-poor (prebeta-1) HDL is the preferred acceptor of cell-derived cholesterol? Does HDL-mediated efflux require HDL binding to the cell surface? And is HDL-mediated efflux linked to a particular membrane cholesterol pool?

Published studies on HDL-mediated efflux are summarized in **Table 2** (refs. 49, 87–93). The results fall sharply into two groups. Mass–balance studies and short-term isotope studies both showed a high degree of specificity for LpA-I-only and lipid-poor prebeta-1 HDL. On the other hand, the long-term isotope studies showed little or no specificity among HDL. Two reasons could explain the differences found. The prebeta-migrating fraction was removed from HDL by ultracentrifugation (31) prior to its use as cholesterol acceptor in many of the long-term efflux studies; and long-term measurements emphasize the contribution to efflux from slow transfer pools in the plasma membrane. Other data consistent with the existence of fast, prebeta-HDL-dependent efflux (94) and slow "non-specific" (probably diffusional) efflux are shown in **Table 3.** Prebeta-1 HDL-dependent efflux may be from the pool modified by SCP-2 in L-cell membranes, although this needs to be experimentally verified. The activity of this component of efflux (about 60% of total efflux in normal fibroblasts) can also be modified by the rate of LCAT activity (29). It is conveniently assayed in terms of its ability to maintain prebeta-1 HDL levels. This is maintained over at least 90 min into native plasma (95), indicating

TABLE 2. Effects of Lp(A-I) and Lp(A-I,A-II) on cholesterol efflux

Reference	Cell	Fractionation Method	Time Course	HDL Specificity
Mass studies				
49	Fibroblast	I	1 h	LpA-I > LpA-I,A-II
87	Adipocyte	I/C	0.25–24 h	LpA-I > LpA-I,A-II
88	Adipocyte	I or I/C	0.25–24 h	LpA-I > LpA-I,A-II*
89	Macrophage	I	24 h	LpA-I > LpA-I,A-II
Short-term isotope studies				
29	Fibroblast	E	1 min	LpA-I > LpA-I,A-II
90	Fibroblast	E	1 min	LpA-I > LpA-I,A-II
Long-term isotope studies				
91	Hepatoma cell, fibroblast, smooth muscle cell	M + I	0.5–24 h	LpA-I = LpA-I,A-II
92	Macrophage	C + I	1–20 h	LpA-I = LpA-I,A-II
93	Fibroblast, endothelial cell	C + I	6 h	LpA-I = LpA-I,A-II

I, immunoaffinity chromatography on native plasma without ultracentrifugal flotation; I/C, immunoaffinity chromatography and ultracentrifugal flotation; E, two-dimensional nondenaturing electrophoresis; C + I, initial ultracentrifugal flotation followed by immunoaffinity chromatography. M + I, molecular sieve + immunoaffinity chromatography.
*More effective mass efflux was found when plasma was not centrifuged prior to immunoaffinity chromatography.

TABLE 3. Properties of specific (prebeta-HDL dependent) and nonspecific (independent) cholesterol efflux

Specific Efflux	Diffusional Efflux
1. Fast pool ($t_{1/2}$ ~ 1 h)	1. Slow pool ($t_{1/2}$ ~ 10 h)
2. Protease-sensitive	2. Protease-resistant
3. LpA-I-dependent	3. LpA-I = LpA-I,A-II; also to albumin
4. Maintains prebeta-1 HDL	4. Does not maintain prebeta-1 HDL levels
5. Linked to LCAT-mediated esterification	5. LCAT-independent

that this pool is not depleted with time. Unlike nonspecific efflux the prebeta-HDL component is protease-sensitive (94). Prebeta-HDL-dependent efflux seems to be completely absent in erythrocytes. The whole of efflux from these cells may be diffusion-based.

The model shown in **Fig. 5** extends that proposed by Rothblat and colleagues (8) in the following ways. Two components are now believed to contribute to total efflux: prebeta-1 HDL-dependent (specific) and diffusion-based (nonspecific). In the specific pathway, cellular lipid transfer proteins (FABP, SCP-2, and an unidentified cytoplasmic or lysosomal protein (96) are possible candidates) may regulate the size and properties of the fast pool of cell membrane cholesterol that interacts with prebeta-1 HDL, via effects on the distribution and kinetic properties of cholesterol on the outer leaflet of the bilayer. The unique conformation of apoA-I in prebeta-1 HDL may define a sterol-binding site. Cholesterol transfers from the membrane to the bound prebeta-1 HDL, inducing a conformational change that promotes desorption from the membrane surface. The cholesterol transferred is retained in prebeta-1 HDL, probably because sphingomyelin reduces its off-rate, as it does in synthetic membrane surfaces (97). This model, while still tentative, is consistent with the recent data summarized in Table 2 and Table 3.

In native plasma albumin may be an important acceptor of the diffusional component of total efflux. Its relatively small diameter (compared to most plasma lipoproteins) and ability to complex cholesterol make it an attractive candidate for initial acceptor of the diffusional or nonspecific component of efflux. From this complex the cholesterol could diffuse to other lipoproteins such as alpha-migrating HDL and LDL, although a smaller proportion of direct transfer to these lipoproteins may also occur. When all of apoA-I was removed from plasma by immunoaffinity chromatography, total efflux was decreased to about 50%. The residue was mainly albumin-dependent (98).

One laboratory recently reported that isotope from cholesterol-labeled cells was partly recovered on an apoE-rich lipoprotein lacking apoA-I or apoB (99) and migrating with a slow gamma-mobility. An acceptor with these properties had been identified earlier in the plasma of noninsulin-dependent diabetics, but the particle could not be detected in normal plasma (100), and no apoE antigen in the gamma position could be found (77). Finally, when total apoE (unlike total apoA-I) was removed from plasma by immunoaffinity chromatography, there was no reduction in cholesterol efflux from fibroblast monolayers (49). An apoE-only lipoprotein would be relatively ineffective

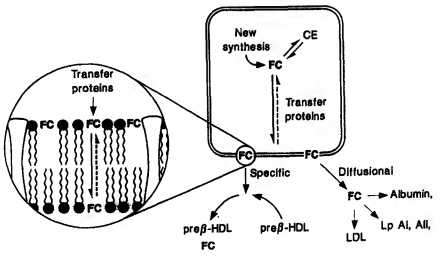

Fig. 5. A model for heterogeneous cellular cholesterol efflux. LpAI,AII, HDL containing both apoA-I and apoA-II. Other abbreviations are as in Fig. 1.

as cofactor in the LCAT reaction (57) and, in fact, removal of apoE from plasma also had no effect on plasma LCAT activity (49). These data taken together cast doubt on a general role for an apoE-particle in RCT, at least in normal plasma. The proportion of cell-derived cholesterol identified in alternative (non-apoA-I) pathways in general may depend on such variables as total HDL concentration, cholesterol-loading of cells, and the time-course of the study, as well as the identity of the cell. The possibility that HDL proteins other than apoA-I (such as apoE) play a more major role in interstitial fluid has not been excluded; and in pathological plasma samples, the contributions of different efflux pathways may be quite different from those in normal physiology.

Transfer of cholesterol in prebeta-1 HDL to discoidal or prebeta-2 HDL

Labeled cholesterol newly transferred to prebeta-1 HDL from the cell surface rapidly appears in prebeta-2 HDL with precursor-product kinetics (29, 101). The increased proportion of these particles in interstitial fluid suggests that an important part of this reaction probably occurs when HDL, or apoA-I dissociated from it, cross the vascular bed. The implications of several alternative mechanisms need to be considered.

In the first, lipid-poor HDL fuse with each other or with preformed small discoidal HDL as lecithin is transferred to the particle. CHO cells transfected with human apoA-I initially secrete lipid-poor HDL. Later larger probably discoidal particles are observed but with a long (8–24 h) lag period (102). PLTP may increase the rate of disc formation but experimental data indicating a role for PLTP in RCT is still lacking. PLTP mRNA is enriched in the endothelium (74). Alternatively, all the lecithin required may be transferred from cell membranes to prebeta-1 HDL as these particles increase in diameter, using the same mechanism as mediates cholesterol transfer (95). In both these models apoA-I remains with the enlarging disc during LCAT activity, until lost from the mature spheroidal HDL product in a reaction discussed later (**Fig. 6**). Consistent with this concept, the vascular bed contains the increased concentration of prebeta-1 HDL expected if this was generated locally (34, 35). As would be predicted, lymph also contains an increased concentration of both prebeta-1 HDL and discs (32, 33). LCAT activity is inhibited in lymph owing to an unusual conformation of apoA-I in its discoidal HDL population (103). Enlargement of the disc would continue as cholesterol and lecithin were transferred from parenchymal cells to generate the optimum LCAT substrate, large HDL discs (12–14 nm diameter) containing three to four apoA-I (104, 105). When the lymphatic HDL enter the plasma, LCAT activity begins, as discoidal HDL are present in plasma only at very low concentrations. The process by which lymphatic apoA-I is changed to a conformation reactive with LCAT is not yet known; most likely the lipid

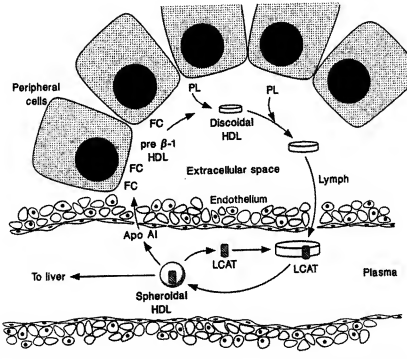

Fig. 6. The synthesis of discoidal HDL in extracellular fluid. PL, phospholipid; LCAT, lecithin:cholesterol acyltransferase. Other abbreviations are as in Fig. 1.

composition of the disc changes when it becomes exposed to plasma concentrations of the major lipoprotein fractions. The model in Fig. 6 includes the features described.

An alternative mechanism would have prebeta-1 HDL as a cholesterol transfer protein between peripheral membranes and preformed discoidal HDL, recycling to the cell surface before further metabolism of the disc and in particular, before any LCAT-mediated esterification of cell-derived cholesterol. This hypothesis is unsupported by experimental evidence, but cannot be completely ruled out at this time.

Binding of LCAT to discoidal HDL

Native discoidal HDL are by far the most effective substrate for LCAT (40). Why does LCAT bind preferentially to discoidal HDL, and why is cholesterol the preferred acceptor of lecithin-derived acyl groups?

The central region of apoA-I (approximately residues 99–186, Fig. 2) clearly plays a key role (**Table 4**, refs. 43, 44, 105–107). Within the central region the strongest data involve the conserved repeat spanning residues 143–165 of mature human apoA-I and the two poorly conserved segments on either side. Deletion of the 143–165 repeat completely inhibited the activation of LCAT by apoA-I. A second locus, possibly of less importance, involves residues 88–121. When this region was deleted, activation by apoA-I was reduced by 70–80%. Monoclonal antibodies reduced activation by 40–90%.

The conclusion that a specific repeat in apoA-I plays an essential role in the LCAT reaction may seem at first sight inconsistent with data showing that short peptides unrelated to apoA-I can activate LCAT (27). This need not be the case, if the functional specialization implied by the data in Table 4 represents a response to the large size and multi-repeat structure of native apoA-I, compared to a small polypeptide with similar amphipathic helical structure.

Residues 121–142 and 166–187 are class A1 helices (26) but unusual in their low hydrophobic moment/residue (0.23 in each case vs. an average of 0.34 for apolipoprotein

TABLE 4. Effects of deletion or neutralization of apoA-I repeating sequences on activation of LCAT

Reference	Technique	Sequence	Inhibition
			%
43	Mutagenesis	148–186	100
44	Mutagenesis	140–164	98
		165–186	98
106	mAb neutral	95–121	80
105	mAb neutral	96–122	77–98
		135–148	56–85
		149–186	37–91

The monoclonal antibody (mAb) that inhibited LCAT activation on synthetic recombinant discoidal HDL was also effective in inhibiting cholesterol efflux by cultured monocytic cells to the same particles (ref. 107). Ranges given in ref 105 refer to discoidal HDL of different compositions.

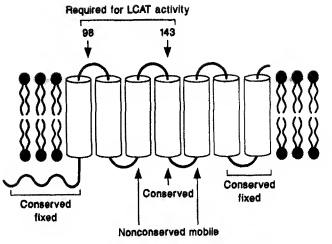

Fig. 7. ApoA-I structure in discoidal HDL: the sliding repeat model. Repeats correspond to those identified in ref. 41.

repeats in general). Their hydrophobic residues (mainly leucines) are constricted into an unusually narrow arc. There is no clustering of charged residues. Together these data suggest that residues 121–142 and 166–187 interact relatively weakly both with the phospholipid acyl chains exposed at the periphery of the disc and with adjacent apoA-I repeats. Residues 143–166 represent a more typical class A1 helix with a hydrophobic moment of 0.39/residue. It contains a grouping of positive charges that is highly conserved between species. At each end of the apoA-I polypeptide are more hydrophobic repeats. If these anchor the N- and C-termini in place on the disc periphery, adjustment to changes in disc diameter will occur mainly by lateral movement of residues 121–142 and 166–187. The short synthetic polypeptides, (typically with two amphipathic helical repeats) which both induce HDL disc formation and activate LCAT, should be able to move laterally much more readily. In **Fig. 7** the N- and C-terminal thirds of the molecule are shown anchored in the disc periphery, while the middle third contains the two weakly interactive "sliding" repeats and the conserved middle domain.

The same mechanism may also explain binding of LCAT to the disc. Like residues 143–165 of apoA-I, the apoE-like sequence of LCAT (residues 151 and 174) has a hydrophobic moment normal for a class A1 amphipathic helix. The pattern of charged residues in the helices of apoE and LCAT is very similar (**Fig. 8**) except for the substitution of a glu residue (E_{166} in LCAT) for a lysine (K_{282}) in apoE. Several negative charges (glu_{154}, glu_{155}, glu_{165}, glu_{166}) are located near the interface of hydrophobic and hydrophilic faces. If the distribution of charges in residues 143–165 in apoA-I and 151–174 in LCAT is compared, many of the positive charges of one seem to match negative charges on the other. Charge–charge interaction between LCAT and residues 143–165 of apoA-I, suggested

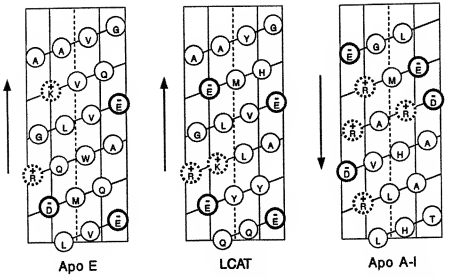

Fig. 8. Helical net representation of apoE residues 268–288, LCAT residues 152–172, and (antiparallel) apoA-I residues 144–163. Negatively charged residues are identified by heavy circles, positively charged residues by discontinuous circles, and uncharged residues by light circles.

by this, is also implied by the quantitative dissociation of the enzyme from HDL in concentrated salt solutions (57).

In addition to its activation by apoA-I and preference for discoidal HDL, LCAT has a third unusual feature: its specificity for cholesterol as acyl acceptor. Free cholesterol must obviously be present equimolar with lecithin at the point where LCAT active site residues interact with substrate; the molar ratio of cholesterol relative to lecithin can be < 0.10 yet transesterification remains almost quantitative (108). The C-terminus of apoE is believed to represent that part most involved in lipid binding by this polypeptide (47, 109). The conformation of residues 268–288 in apoE is very similar to that present in LCAT between residues 151–174. Based on this similarity, we suggest that residues 151–174, in addition to their proposed reaction with apoA-I, serve as a sterol binding domain, drawing cholesterol to the periphery of the disc for transesterification adjacent to active site $serine_{181}$. A molecular model for the interaction of LCAT with discoidal HDL in shown in **Fig. 9.** Residues 151–174 of LCAT bind antiparallel to residues 143–165 of apoA-I, orienting the two substrates at the edge of the disc. Short beta-sheet sequences form two sides of a basket containing ser_{181}.

Further reaction of LCAT with HDL

Following the initial reaction of LCAT with HDL discs, intermediate lens-shaped particles have been identified (110). As the sphere is formed there will be displacement of adjacent apoA-I repeats as the polypeptide extends over the surface (Fig. 4). The affinity of LCAT for the HDL surface must decrease with increasing HDL diameter, because large HDL contain little if any LCAT (111, 112).

Several catalyzed reactions can generate free or lipid-poor apoA-I from spheroidal HDL in vitro. Hepatic lipase also generated small prebeta-1 HDL from alpha-migrating particles during perfusion through the isolated liver (113). The addition of isolated hepatic lipase to native plasma was required to generate these small HDL, even though the plasma contained CETP (114). In another study CETP could generate prebeta-HDL from either LpA-I or LpAI,AII fractions of alpha-HDL, but a many-fold excess of CETP over physiological levels (relative to HDL) was used (115). Prebeta-HDL is present at concentrations similar to those in human plasma in mice (which have hepatic lipase but lack plasma CETP) while it was undetectable in rabbits (P. E. Fielding, unpublished experiments) which have high levels of CETP but little hepatic lipase. Hepatic lipase appears the most likely candidate to

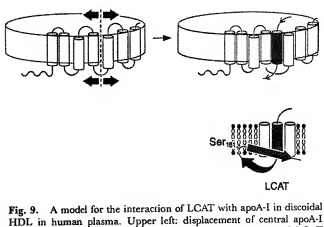

Fig. 9. A model for the interaction of LCAT with apoA-I in discoidal HDL in human plasma. Upper left: displacement of central apoA-I repeats; upper right, insertion of the apoE-like segment of LCAT (shaded cylinder) across the bilayer. Lower right: alignment of the active site region. Beta sheet is shown on each side of active site ser181.

generate the bulk of prebeta-1 HDL from alpha-HDL in human plasma, although further research is needed.

The mechanism of reaction would be the same for hepatic lipase or CETP. Both can decrease HDL core lipids, leaving "excess" surface (mainly phospholipids and apoA-I) available for dissociation or transfer. An increase in prebeta-HDL postprandially has been reported, particularly after heparin injection (116) when plasma activities of hepatic lipase and LCAT and CETP activity are increased (117). Lipid-poor HDL would be available for filtration through the vascular bed, completing the cycle shown in Fig. 6. Whether LCAT dissociate from the enlarging HDL as a result of the same remodelling process or whether product inhibition plays a more important role (118) is not yet settled.

Lipid-free apoA-I interacts effectively with cholesterol-loaded macrophages, removing cholesterol and phospholipid and generating prebeta-migrating HDL with a composition similar to that of the native prebeta-1 HDL particles recovered from human plasma (119). In smooth muscle cells, cholesterol efflux is less but phospholipid transfer is maintained (120). These data suggest that a precursor of native prebeta-1 HDL may be apoA-I transferred lipid-free (or nearly so) from spheroidal HDL.

LpA-I and LpA-I,A-II in reaction with LCAT

All the data reviewed in this section so far indicate that HDL containing only apoA-I (LpA-I) play the major role in RCT. If this is so, what then is the function of the LCAT bound to LpA-I,A-II particles, the majority of which make up the alpha-migrating HDL fraction? These particles are the initial acceptors for the major part of free cholesterol transferred from LDL in response to plasma LCAT activity (121). Kinetically, interconversion of LpA-I and LpA-I,A-II in the circulation appears to be quite incomplete (122). Consistent with this, cholesterol derived from cells and LDL remains compartmentalized in plasma. The esterification of free cholesterol transferred from LDL took place almost completely within the alpha-migrating HDL fraction (111).

LpA-I,A-II are secreted from cultured hepatoma cells (123). In fact, the liver may be the only significant primary source of these particles (124) which are the likely precursors of much of the alpha-migrating, LpA-I,A-II HDL in plasma. The rate of secretion of apoA-II by the hepatocyte may be an important determinant of the proportions of LpA-I and LpA-I,A-II HDL in plasma. LCAT binding to spherical LpA-I,A-II particles is unlikely to require the same alignment of LCAT residues 151–174 as required for the RCT pathway. The proportion of LpA-I and LpA-I,A-II HDL may, therefore, be determined by the rate of secretion of apoA-II from the liver. These data suggest that the metabolism of cell- and LDL-derived cholesterol may remain largely distinct in the circulation, utilizing LpA-I and LpA-I,A-II, respectively (**Fig. 10**).

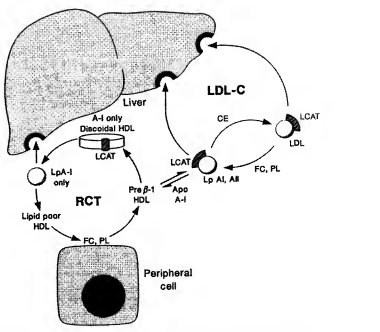

Fig. 10. The metabolism of LpA-I and LpA-I,A-II in human plasma. RCT, reverse cholesterol transport cycle; LDL-C, metabolism of LDL FC. A small proportion of LCAT is also found directly associated with LDL in normal plasma.

One laboratory has recently suggested that the effect of LCAT in RCT is merely a sparing action, without effect on efflux (125). This is not compatible with unloading of cholesterol mass observed in a variety of cell experiments (Table 2). The other data reviewed above suggest that, on the contrary, specific amino acid sequences in LCAT and apoA-I play a major directing role in RCT and the utilization of cell free cholesterol.

CETP activity

Data of several kinds indicate that CETP does not play a major role in RCT, at least in normal metabolism. Inhibition of CETP activity in normal plasma with neutralizing monoclonal antibodies did not inhibit LCAT activity (126). Little (about 5%) of the labeled cholesteryl ester generated by LCAT activity with [^{3}H]cholesterol from labeled cells was transferred to VLDL and LDL (111). From these data it seems most likely that CETP (like LpA-I,A-II) is involved mainly in the metabolism of VLDL- and LDL-derived lipid and not with RCT; although it is possible that changes in CETP activity, or of its acceptors VLDL and LDL, may themselves affect the distribution of LCAT between LpA-I and LpA-I,A-II.

Clearance of cell-derived cholesterol by the liver

There are still little or no data at the molecular level to explain the recognition of HDL of different kinds. Cholesteryl esters in LpA-I are removed by the liver and processed into bile more effectively than those derived from

LpA-I,A-II (127). This may reflect, at least in part, the more effective binding of LpA-I-HDL to the hepatocyte membrane (128).

EVIDENCE FOR RCT IN VIVO

Evidence for the mechanism of RCT discussed above has been drawn almost exclusively from cultured cells and plasma or purified lipoproteins, enzymes, and transfer proteins. The model proposed is a relatively simple one: cell-derived cholesterol is initially bound to lipid-poor, prebeta-migrating HDL; by addition of further lipids (particularly phospholipid) a discoidal, lipid-rich particle is formed, which reacts with LCAT; reaction of cell-derived cholesterol with LCAT prevents its re-entry into the cell. How good is the evidence that RCT occurs like this in vivo, as part of whole body cholesterol homeostasis?

Genetic diseases affecting proteins in the RCT pathway

Because of redundancy within the apoA-I, apoA-IV, apoE family, genetic deficiency of apoA-I seemed unlikely to block RCT. This prediction was confirmed with apoA-I-knockout mice (129). On the other hand, congenital LCAT deficiency is associated with the widespread accumulation of cholesterol in peripheral tissues, particularly the vessel walls, kidney, and spleen. The plasma accumulates prebeta-1 and discoidal HDL, consistent with the postulated precursor role of these particles in the LCAT reaction affecting RCT (29). LDL in LCAT deficiency is low and abnormal, explaining the absence of coronary artery disease in LCAT deficiency.

Fish-eye disease (FED) is mediated by mutations in LCAT that affect the ability of the enzyme to react with HDL ($1.063 < d < 1.21$ g/ml) ("alpha-LCAT activity") but have little or no effect on LCAT activity in vitro when free cholesterol substrate for LCAT ("beta-LCAT activity") is supplied by LDL (130). Possibly the substrate for LCAT in FED plasma is an apoA-I-LDL complex such as observed at low concentration in normal plasma. Based on the model shown in Fig. 10, mutants affecting the conformation of residues 151–174 of LCAT may be among those giving rise to fish-eye disease. The concentration and activity of prebeta-HDL in FED have not been reported.

In congenital CETP deficiency, large HDL accumulate but cholesterol does not accumulate in the tissues (4). This is consistent with the minimal role of CETP in RCT proposed in this review. On the other hand, congenital hepatic lipase deficiency is associated with accelerated atherosclerosis (131), consistent with its role in the generation of prebeta-1 HDL.

Modulation of RCT in vivo

Rabbits injected intravascularly with acetylated LDL accumulate cholesterol in arterial foam cells (132). After disappearance of the circulating acetylated LDL from plasma there was a sustained increase in the HDL cholesterol fraction during the regression phase. Hepatectomy in rats blocked the removal of RCT-derived lipoprotein cholesterol (133). After hepatectomy there was a significant rise in HDL cholesteryl ester. When plasma LCAT activity was inhibited, this increase in HDL cholesteryl ester was blocked. Similar studies have been carried out with freshly isolated spleen or spleen–liver preparations, where a net mass transfer of cholesterol from the spleen to plasma as cholesterol was esterified was observed (134). These data are consistent with the primary role of HDL as acceptor of cell-derived cholesterol, and illustrate the LCAT-dependent mass transfer of cholesterol from tissues to HDL.

RCT in transgenic mice

Highly informative experiments on the mechanism of RCT have been carried out in transgenic mice by several laboratories. These illustrate, in particular, the early effects of apoA-I and the relationship between LpA-I and LpA-I/A-II HDL. There have been no transgenic studies of cellular proteins potentially involved in cholesterol efflux. Mice have a prebeta-HDL that appears similar in properties to that in human plasma (135). In mice overexpressing human apoA-I, mouse apoA-I concentration is greatly reduced. HDL in the circulation of these animals contains mainly human apoA-I and assumes its characteristic human size distribution (136, 137). The concentration of prebeta-migrating particles was increased (137). As a result, transgenic mice appear to be ideal models for studying human RCT in vivo.

As a key concept of the RCT hypothesis from in vitro studies is that lipid-poor prebeta-HDL can act as a major acceptor of cell-derived cholesterol, the concentration of these particles in plasma in vivo should reflect the rate of cholesterol transport from cells to their medium. Atherogenesis (in mice, in response to cholesterol-rich diets in susceptible strains) represents an effective challenge to the hypothesis, as the accumulation of tissue cholesterol is extreme and occurs adjacent to the plasma compartment. Mice with a high concentration of small, lipid-poor prebeta HDL should be protected against cellular cholesterol accumulation, while mice in which small HDL were reduced or absent should be susceptible. This prediction has recently been verified in several different studies.

Mice overexpressing human apoA-I were protected against the atherogenic effect of diets rich in saturated fat and cholesterol (138). When mice were made transgenic for apoA-II the small, lipid-poor fraction of HDL disappeared (139). At the same time the animals became oversensitized to dietary cholesterol (140, 141). When the proportion of LpA-I only and LpA-I/A-II HDL was varied with total HDL kept constant, animals in which apoA-I-only HDL predominated were protected against aortic

cholesterol accumulation, while those in which apoA-I/A-II HDL predominated were most at risk (142). Overexpression of apoA-I in mice also significantly decreased lesion formation in atherosclerosis-prone, apoE-deficient mice (143).

Atherosclerosis in these mice is limited to early (fatty streak) events. However, these are believed to be the precursors of most or all of the complex lesions developing over time in human disease. Unfortunately, some studies of transgenic mouse HDL have used the centrifugal d< 1.20 g/ml or d<1.21 g/ml supernate of plasma for characterization, from which the small prebeta-HDL fraction would be quantitatively lost (31). Nevertheless, where data are available, they appear consistent with the concept that prebeta-HDL concentration in plasma reflects RCT, and that prebeta-HDL can be protective, at least in part, against atherogenesis. Of course, in some situations prebeta-HDL, even though increased in activity, may mitigate but not prevent tissue cholesterol accumulation; and whether the process works in humans as it does in transgenic mice still remains to be determined.

Overall, the results of in vivo studies appear reasonably consistent with predictions based on studies of RCT in vitro. In particular, the special role of prebeta-migrating LpA-I HDL is supported (Fig. 7), as well as the concept that HDL containing both apoA-I and apoA-II are relatively ineffective in promoting RCT (Fig. 10). Further structure/function analysis of key intermediates of RCT will refine our understanding of this complex but important pathway.

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